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Probing the dynamics of intact cells and nuclear envelope precursor membrane vesicles by deuterium solid state NMR spectroscopy

Marie Garnier-Lhomme [a,b], Axelle Grélard [b], Richard D. Byrne [a], Cécile Loudet [b], Erick J. Dufourc [b,*], Banafshé Larijani [a,*]

[a] *Cell Biophysics Laboratory, Lincoln's Inn Fields Laboratories, Cancer Research UK , 44, Lincoln's Inn Fields, WC2A 3PX London*
[b] *UMR 5248 CNRS-Université Bordeaux 1-ENITAB, IECB, 2 rue Robert Escarpit, 33607 Pessac Cedex London*

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Abstract

Membrane dynamics is an essential part of many cellular mechanisms such as intracellular trafficking, membrane fusion/fission and mitotic organelle reconstitution. The dynamics of membranes is dependent primarily on their phospholipid and cholesterol composition and how these molecules are ordered in relation to one another. To determine the physical status of membranes in whole cells or purified membranes of subcellular compartments we have developed a novel application exploiting solid-state ^{2}H-NMR spectroscopy. We utilise this method to probe the dynamics of intact sperm and nuclear envelope precursor membranes. We show, using mass spectrometry, that either multilamellar or small unilamellar vesicles of deuterium-labelled palmitoyl-oleoylphosphatidylcholine can be used to probe the dynamics of sperm cells or nuclear envelope precursor membrane vesicles, respectively. Using ^{2}H-NMR we determine the order parameters of sperm cells and nuclear envelope precursor membrane vesicles. We demonstrate that whole sperm membranes are more dynamic than nuclear envelope precursor membranes due to the higher cholesterol levels of the latter. Our new application can be exploited as a generic method for monitoring membrane dynamics in whole cells, various subcellular membrane compartments and membrane domains in subcellular compartments.

Keywords: Nuclear envelope membrane; Cholesterol; Solid-state NMR spectroscopy; Membrane dynamics

1. Introduction

The complex nature of natural membranes is dependent on their lipid composition. The cell membrane and the various membranes in sub-cellular compartments are "soft matter" and their anisotropic properties are essential for their function. The proteins and lipids that are embedded undergo many dynamic processes (lateral and transverse diffusion) thus by essence the membrane is a dynamic structure. There is considerable evidence that membrane lipid composition and membrane molecular order has a major effect on the function and properties of proteins that associate to the membrane and on the intrinsic proteins [1–3]. Membrane lipid composition is complex, spatially heterogeneous and modulates changes of fluidity in the bilayer environment. In recent years there has been much debate on the means by which natural membranes can be probed accurately to determine their local structure and dynamics. Different methods such as electron spin resonance, cryo-electron microscopy, differential scanning calorimetry (DSC) and fluorescent membrane probes have been used to investigate the physical status and dynamics of membranes.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; $\Delta\nu_Q$, quadrupolar splitting; ESI-MS/MS, electrospray ionisation tandem mass spectrometry; HPLC, high pressure liquid chromatography; MLV, multilamellar vesicle; MV0, precursor egg membrane vesicles; PtdAc, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; POPC, palmitoyl-oleoylphosphatidylcholine; S10, activated egg cytoplasm; S_{CD}, order parameter; ssNMR, solid-state NMR; SUV, small unilamellar vesicles; TEM, transmission electron microscopy

* Corresponding authors. E.J. Dufourc is to be contacted at tel.: +44 207 269 3082; fax: +44 207 269 3094. B. Larijani, tel./fax: +33 5 4000 2218.
E-mail addresses: e.dufourc@iecb.u-bordeaux.fr (E.J. Dufourc), banafshe.larijani@cancer.org.uk (B. Larijani).

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However, most of these techniques, as well as being invasive, do not directly measure the fluidity of the membrane. For instance both fluorescent probes and spin-labelled probes modify the original state of the membrane and DSC does not directly measure the molecular order but measures the heat changes that accompany lipid phase transitions [4].

Solid-state ^{2}H-NMR spectroscopy is one of very few non-invasive methods that reports on ensemble properties and on time and space averaged orientations of molecules or molecular bonds in lipid bilayers. The parameter depicting these properties is known as the order parameter, S_{CD}. ^{2}H-NMR spectroscopy determines the nature of various lipid phases (e.g. lamellar, hexagonal, cylindrical, cubic, etc.) as well as the min dynamics of deuterated lipid molecules in membrane bilayers. In turn, dynamics can be converted into information describing the physical characteristics of membranes, i.e., membrane thickness [5–9] and membrane protrusions [10–13]. Physical parameters such as membrane thickness and membrane fluidity are directly associated with the fusogenity and the mobility of the membrane. Solid-state ^{2}H-NMR has been extensively used to study the dynamics of model membranes and the role of cholesterol in ordering membranes [3,14,15]. There are very few studies that have investigated microfluidity in natural membranes. In one of these studies deuterated labelled fatty acid chains were incorporated in the cellular growth medium of Gram-positive bacteria *Acholeplasma laidlawii*. The effect of microfluidity was investigated on the extracted lipids [16,17]. Moreover, the influence of cholesterol on the order parameter was monitored using the above method [18] or by exchange techniques in human red blood cells [19]. In other studies, the lamellar structural stability of membranes reconstituted from extracted *A. laidlawii* or *Escherichia coli* lipids was investigated using heavy H_2O [20,21].

Nonetheless these methods were limited to reporting information on extracted lipids and they did not probe the dynamics of intact cells or purified membrane compartments directly.

To directly monitor the dynamics of intact cell membranes or subcellular membrane vesicles, sea urchin sperm and nuclear envelope precursor membrane vesicles from fertilised sea urchin cytoplasmic egg extracts were labelled with saturated 16:0/16:0 (DPPC-$^2H_{62}$) and unsaturated 16:0/18:1 (POPC-$^2H_{31}$) ^{2}H-labelled phosphatidylcholine (PtdCho). These specific biological samples were chosen because of their purity and abundance. We determined the order parameter of intact sperm cells and nuclear envelope precursor membrane vesicles labelled with MLVs and SUVs respectively of DPPC-$^2H_{62}$ or POPC-$^2H_{31}$. Moreover, we optimised the quantity of probe required for labelling whole sperm cells and membrane vesicles by HPLC-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS/MS). Electron and fluorescence microscopy were used to ensure that the membrane structure of whole cells remained intact. Colorimetric assays were used to determine the cholesterol content of sperm cells and nuclear envelope membrane vesicles. The order parameters obtained by NMR that result usually from the presence of cholesterol were compared to model membranes containing various amounts of cholesterol. We show that sperm membrane is ordered and the nuclear envelope membrane vesicles have a higher order parameter due to their greater cholesterol content.

2. Materials and methods

Buffers: SXN: 50 mM HEPES, 50 mM Sucrose, 150 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 300 mM glucose, pH=7.2; MWB: 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 50 mM HEPES, 1 mM DTT, 1 mM ATP, 1 mM fresh PMSF, pH=7.5; Freezing solution: 10 ml SXN+6 ml glycerol+2 ml of 3% BSA in SXN; Reagents: 1,2-Dipalmitoyl-$^2H_{62}$-*sn*-Glycero-3-Phosphocholine (DPPC-$^2H_{62}$) and 1-Palmitoyl-$^2H_{31}$-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC-$^2H_{31}$) from Avanti Polar Lipids, Inc. (USA); Deuterium depleted water was provided by Isotec member of Sigma-Aldrich family ; β-BODIPY 500/510 C_{12}-HPC from Invitrogen; *S. purpuratus* were provided by Marinus Scientific Inc., Long Beach, California, (USA) and *P. lividus* from M^r Tripolitsiotis, Naoussa Paros, 84401 Cyclades (Greece).

2.1. Nuclear envelope membrane precursors (MV0) and sperm suspension

MV0 was isolated from sea urchin fertilised egg cytoplasm (S10) and prepared as previously described [22]. Mature eggs were resuspended in 5 volumes Millipore sea water (MPSW) pH 5.0. Residual acidic MPSW was removed by 2 washes in MPSW. Approximately 10 ml of eggs were resuspended in 100 ml MPSW containing freshly made 3 mM ATA. Fertilisation of the eggs was achieved with sperm diluted in MPSW to give an approximate final sperm:egg ratio of 10:1. Two minutes after fertilisation, eggs were filtered through a 100 μm Nytex filter and washed twice at 100×*g* for 1 min, in 10 volumes of MPSW. This was followed by 3 washes at 4 °C in 10 volumes of ice-cold LB buffer (10 mM HEPES, 250 mM NaCl, 5 mM $MgCl_2$, 10 mM glycine, 250 mM glycerol, 1 mM DTT, pH 8) supplemented fresh with 1 mM PMSF. Eggs were finally resuspended in 1 volume of LB and homogenised by twice vigorously drawing the suspension up through a 22-gauge needle into a 10-ml syringe, followed by forced expulsion. The homogenate was centrifuged at 10,000×*g* for 10 min at 4 °C. Egg cytoplasmic extract was isolated from the supernatant (referred to as S10 hereafter). 1 ml of S10 was centrifuged at 150,000×*g*, 4 °C for 3 h. The resulting pellet containing total membranes (MV0) was resuspended in 1.2 ml MWB (250 mM sucrose, 50 mM KCl, 50 mM HEPES, 1 mM DTT, 1 mM ATP, 1 mM PMSF, pH 7.5) and centrifuged through a sucrose cushion to allow better resuspension. 1.2 ml MWB containing 0.5 M sucrose was added to the bottom of the tube and centrifuged at 60,000×*g*, 4 °C for 15 min. The supernatant was further centrifuged at 60,000×*g* for 15 min at 4 °C. Membrane vesicle pellets were resuspended in 100 μl MWB, snap-frozen in liquid nitrogen and stored at −80 °C. The sperm was concentrated by centrifugation at 500×*g* for 10 min at 4 °C. 250 μl of concentrated viable sperm cells were resuspended in 10 ml of ice-cold SXN buffer in a 15 ml centrifuge tube using a plastic transfer pipette. Sperm was centrifuged at 2600×*g* in a Centra MP4R swinging bucket centrifuge for 5 min at 4 °C. The supernatant was aspirated and the pellet resuspended in 500 μl SXN supplemented by 500 μl of freezing solution. Sperm suspension was snap frozen in liquid nitrogen and stored at −80 °C.

2.2. Electron microscopy of MV0 and sperm

For transmission electron microscopy (TEM), sperm and MV0 pellets were fixed for 1 h in 0.1 M sodium phosphate buffer (Sorensen's) containing 2.5% (v/v) glutaraldehyde in the presence of 1% (w/v) tannic acid. The pellets were post-fixed in 1% (v/v) osmium tetroxide in 0.05 M Sorensen's buffer for 30 min, washed and dehydrated in ascending ethanol series. The samples were embedded in araldite over 2 days and thin sections of approximately 80 nm thickness cut and observed on a JEOL 1010 TEM. For scanning electron microscopy (SEM), pellets were resuspended in 4% paraformaldehyde. A drop was bound to a polylysine-coated cover slip,

dehydrated in a critical point dryer, coated with platinum and viewed on a Field Emission SEM-6700.

2.3. Phospholipid and cholesterol/cholesteryl ester concentration determination

Lipids from sperm suspension and MV0 were extracted according to a modified Folch procedure [23]. Briefly, 200 µl of sample were added to 4 ml of chloroform/acidified methanol (2.5:1), probe sonicated for 10 s and filtered through a 0.22 µm pore. 800 µl of 0.2 M K_4EDTA solution at pH=6 were added to induce phase separation. The mixture was centrifuged at 800×*g* for 15 min at 4 °C the organic phase extracted and dried under nitrogen [24]. Phospholipid concentration was determined by indirect measurement of inorganic phosphates liberated from extracted lipids. Lipid pellets were resuspended in 500 µl TN buffer. Samples and standards (K_2HPO_4 in water from 1 to 500 µM) were supplemented with 100 µl of 10 N sulphuric acid and heated for 1.5 h at 200 °C. 100 µl of 72% perchloric acid was added and samples were further heated for 1 h at 200 °C. A solution containing 14 ml distilled water, 2 ml of 6 N sulphuric acid, 2 ml of 2.5% (w/v) ammonium molybdate ($(NH_4)_6Mo_7O_{24},4H_2O$) in water and 2 ml of freshly prepared 10% (w/v) ascorbic acid in water was prepared. 2 ml of this solution were added to the samples, and the samples were transferred to sealed glass tubes and incubated at 50 °C for 30 min. After incubation, samples were pale blue and absorption was read at 820 nm. Cholesterol and cholesteryl ester concentrations were determined from lipid-extracted samples. Lipid pellets resuspended in 5 µl TN buffer and 5 µl of cholesterol standard solutions ranging from 0.3 to 7.8 mM were supplemented with 500 µl of cholesterol liquid stable reagent provided by Thermo Electron Corporation. The solutions were probe-sonicated (Soniprep 150) at power 10 for 3 s and incubated for 5 min at 37 °C. Absorption was taken at 500 nm.

2.4. Cholesterol-containing multi-lamellar vesicle (MLV) standards

The procedure of Aussenac et al. [15] was followed. The deuterium-labelled lipid and cholesterol were resuspended separately in chloroform at 50 mg/ml. The appropriate volumes of each solution were mixed together to obtain the desired lipid:cholesterol molar ratio: 95:5, 85:15 and 70:30 respectively. A minimum volume of 300 µl was agitated vigorously and the chloroform dried under nitrogen gas. The powder was lyophilised twice in distilled water to remove traces of chloroform. It was hydrated at 90% (w/w) in the appropriate buffer (SXN or MWB) prepared with deuterium-depleted water: three freeze/thaw cycles afforded a homogeneous milky suspension of multi-lamellar vesicles. These liposomes were transferred into a 4 mm zirconia rotor (100 µl).

2.5. Deuterium-labelled phospholipid vesicle for labelling natural membranes

MLVs were prepared as described above (136 mM). Small unilamellar vesicles (SUV) were obtained as follows. The deuterium-labelled lipid was hydrated at 98% (25 mM) in the corresponding deuterium-depleted buffer for a minimum final volume of 1 ml. The suspension was transferred into a 5 ml plastic tube and probe sonicated for at least 15 min above the lipid transition temperature (20 °C for POPC-$^2H_{31}$ and 40 °C for DPPC-$^2H_{62}$). The pulse cycles were 4 s sonication with 6-s intervals. SUVs were centrifuged at 10,000×*g* for 10 min to remove any metal residue from the probe that may interfere with NMR measurements. SUV diameter was measured by dynamic light scattering as described bellow and was 55 nm.

2.6. Vesicle size measurement by dynamic light scattering

A minimum volume of 0.5 ml of SUVs or MV0 was transferred into a glass tube and the correlation function acquired at different angles: 45°, 60°, 75°, 90°, 120° and 150° using an ALV/CGS-3 light scatter (Compact Goniometer System). The data were processed with an ALV Correlator Software V.3.0. The viscosity, η, and the refractive index, n, were adjusted to $\eta=1.19$, $n=1.074$ for SXN and $\eta=1.25$, $n=1.345$ for MWB. An average correlation time, τ_c, was measured for each angle and $1/\tau_c$ was plotted against the scattering vector, q^2, according to the equation: $1/\tau_c=Dq^2$ where D is the diffusion coefficient and $q=[4\pi n \sin(\theta/2)]/\lambda$ with θ the scattering angle and λ the laser wavelength (632.8 nm). The hydrodynamic vesicle radius is calculated from a linear fitting of the graph, $1/\tau_c=Dq^2$, using the equation $D=(k_BT)/(6\pi\eta R)$ where k_B is the Boltzman's constant and T the temperature in Kelvin [25,26].

2.7. Incorporation of deuterated lipids in natural membranes

6 ml of frozen *S. purpuratus* sperm were centrifuged at 500×*g*, 4 °C for 20 min and resuspended in 1.2 ml SXN prepared in deuterium-depleted water. MV0 isolated from 7 ml of S10 was resuspended in 700 µl MWB prepared in deuterium-depleted water. Both deuterium labelled phospholipids, 16:0/16:0 DPPC-$^2H_{62}$ and 16:0/18:1 POPC-$^2H_{31}$, were added to obtain various deuterated to natural phospholipid molar ratios. Sperm cells were incubated for 30 min at 40 °C with MLVs of DPPC-$^2H_{62}$ or POPC-$^2H_{31}$, centrifuged at 500×*g* for 15 min at 25 °C and washed twice in 600 µl SXN. The sperm/MLV pellet was resuspended in 300 µl SXN and transferred in a 7-mm NMR rotor (380 µl). The optimised concentrations in natural and deuterated phospholipids were 3.8 mM and 3 mM, respectively, for the initial 1.1:1 molar ratio in deuterated versus natural phospholipid (Table 1). MV0 was incubated with SUVs of DPPC-$^2H_{62}$

Table 1
Experimental conditions and results for deuterated lipid incorporation into natural membranes

Biological sample			DPPC $^2H_{62}$			Centrifugation force (g)[a]	($^2H_{62}$:[b]L) starting	($^2H_{62}$:L) washes	($^2H_{62}$:L) final	Biological membrane recovery (%)[c]
Name	ml	nmol PL	Suspension	ml	nmol PL					
MV0	5	4.0	SUV	1.46	200.0	1,500	28:1	0.5:1	7.4:1	nd
	5	4.0	MLV	1.46	200.0	1,500	28:1	0.4:1	9.1:1	nd
MV0	40	26.3	MLV	0.67	26.1	500	0.6:1	0.2:1	3.6:1	8.2
	40	32.7	MLV	0.67	19.7	1,500	0.3:1	0.1:1	2:1	10.3
	40	23.5	SUV	3.60	19.0	1,500	0.5:1	0.3:1	0.2:1	15.4
	40	21.6	MLV	0.67	17.2	10,000	0.5:1	0.1:1	2.3:1	15.3
	40	**25.9**	**SUV**	**3.60**	**17.9**	**10,000**	**0.4:1**	**0.2:1**	**0.2:1**	**27.7**
Sperm	**160**	**109.9**	**MLV**	**6.16**	**121.0**	**500**	**0.7:1**	**0.4:1**	**0.5:1**	**73.2**
	160	101.4	SUV	33.35	133.7	500	0.9:1	0.5:1	0.2:1	73.6

MV0 and sperm cells were labelled with DPPC-$^2H_{62}$ also referred to as "$^2H_{62}$".
nd: not determined; PL: phospholipid.
[a] Samples were centrifuged for 15 min to wash out excess DPPC-$^2H_{62}$.
[b] L refers to the phospholipids: PtdCho, PtdEth, Ptdns, PtdAc and PtdSer quantified by mass spectrometry and the cholesterol quantified by colorimetric assay. L does not take cardiolipin in account.
[c] Mol percentages were estimated from ESI-MS/MS quantification by comparison of phospholipids and cholesterol in the supernatant and in the pellet. Optimum conditions are highlighted in bold.

or POPC-$^{2}H_{31}$, for 30 min at 40 °C, centrifuged at 10,000×*g* for 15 min at 25 °C. The pellets were washed twice in 500 µl MWB at the same speed. MV0 was resuspended in 300 µl MWB and transferred in a 7 mm NMR rotor (800 µl). The optimised concentrations in deuterated and natural phospholipids were 0.14 mM and 0.47 mM respectively for the initial 0.5:1 molar ratio in deuterated versus natural lipids (Table 1).

2.8. Deuterium solid-state NMR spectroscopy experiments

NMR experiments were carried out at 46 MHz on a Bruker Avance 300 WB (7.05 T) spectrometer for model membranes and at 76.8 MHz on a Bruker Avance DSX 500 spectrometer (11.75 T) for biological membranes. The 300 and 500 spectrometers were equipped with a CP MAS triple 4 mm 1H/X/Y and a static triple WB 1H/X/Y probe, respectively. The triple WB 1H/X/Y probe was equipped with an in house 7 mm coil with 10 turns or a 10 mm coil with 11 turns to increase sensitivity. Spectra were acquired by means of a quadrupolar echo pulse sequence 90°x–τ–90°y–τ–acq [27]. The acquisition parameters for model membranes were as follows: spectral window of 500 kHz, $^{\pi}/_{2}$ pulse width of 4.5 µs, the recycle delay was 1 s, the echo delay that separates pulses was 50 µs. Typically, 1024 acquisitions were recorded. Samples were allowed to equilibrate for 30 min at a given temperature before the NMR signal was acquired. The acquisition parameters for biological membranes were: spectral window of 500 kHz, $^{\pi}/_{2}$ pulse width ranged from 3.5 and 6.5 µs depending on the osmolarity of the buffer and coil diameter, the recycle delay was 1 s and the echo delay 30 µs. The number of acquisitions was dependent on the sample concentration, coil diameter, and ranged from 10 to 90 k. Samples were allowed to equilibrate 45 min at a given temperature before the NMR signal was acquired. The typical experimental temperatures were 20 °C and 40 °C when using DPPC to label natural membranes and 10 °C and 40 °C when using POPC.

2.9. Calculation of order parameter

Carbon-deuterium order parameters, S_{CD}, were calculated from quadrupolar splittings, $\Delta\nu_{Q}$, measured on the most intense peaks (known as 90°

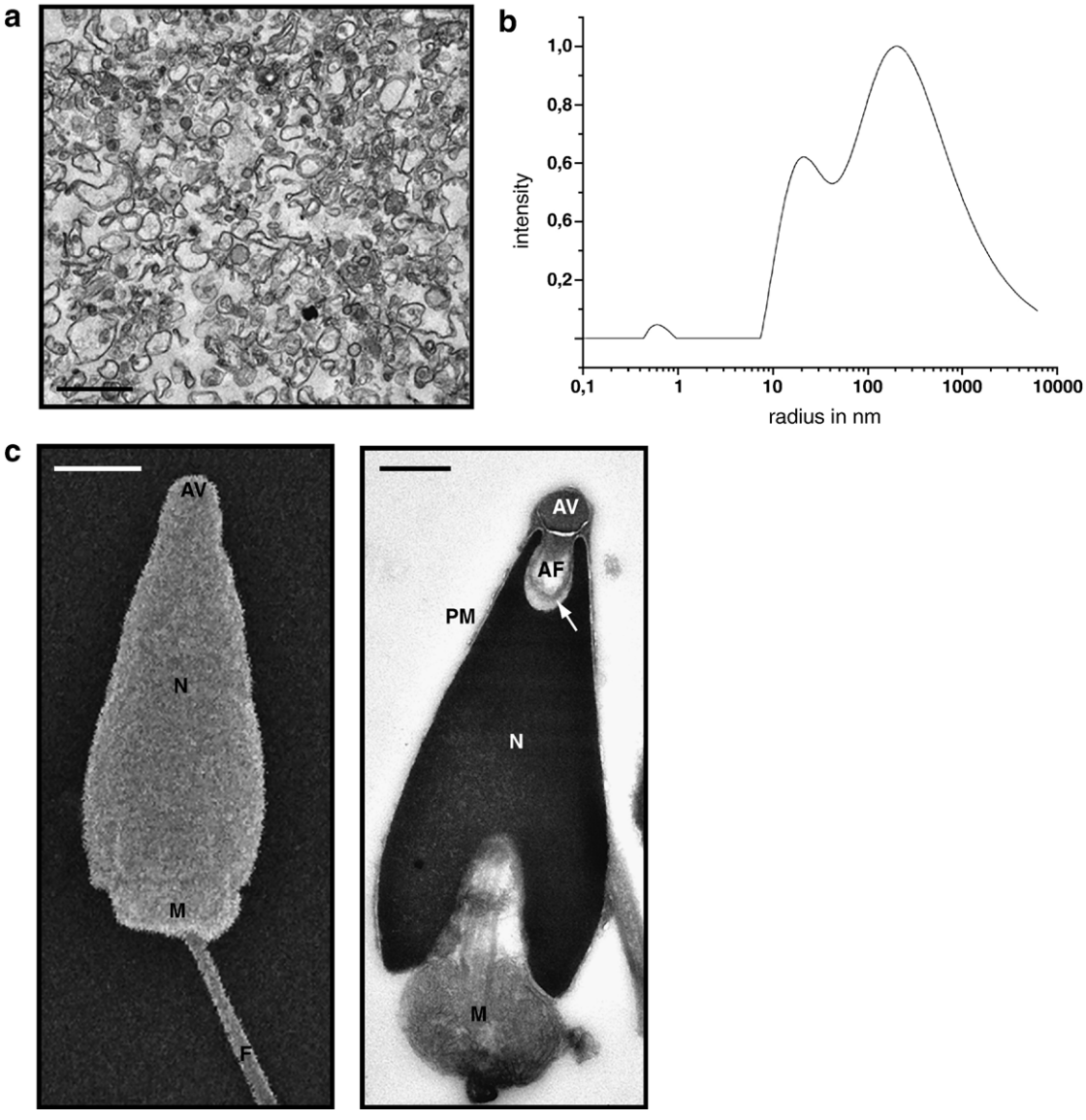

Fig. 1. Membrane vesicle and whole sperm morphologies by electron microscopy and light scattering. (a) Precursor egg membrane vesicles (MV0) were fixed in 2.5% gluteraldehyde, dehydrated, embedded in a resin according to Materials and methods and viewed by transmission electron microscopy (TEM). (c right panel) *S. purpuratus* sperm were pre-fixed in 2% formaldehyde, 0.2% glutaraldehyde, 1% acrolein, cryo-protected, cryo-sectioned and viewed by TEM. (a) MV0 size was between 100 nm and 500 nm by electron microscopy. Bar is 1 µm. (b) MV0 size distribution was determined by dynamic light scattering. (c) Left: scanning electron micrograph of whole *S. purpuratus* sperm fixed in 4% formaldehyde: AV, acrosomal vesicles; N, nucleus; M, mitochondria; F, flagellum. (c) Right: TEM of *S. purpuratus* sperm shows the double bilayer of the nuclear envelope (arrow). AF, acrosomal fossa; PM, plasma membrane. Bars are 500 nm.

orientations) on axially symmetric powder spectra using the following equation:

$$S_{CD} = (4\Delta\nu_Q)/(3A_Q) \quad (1)$$

A_Q is the static quadrupolar coupling constant (167 kHz, [28]). For carbon-deuterium bonds (methylene groups) oriented perpendicular to the lipid long axis (bilayers normal), a geometrical factor 2 must be considered and the quantity $2^*|S_{CD}|=0$ thus indicates that the labelled position of interest is completely disordered whereas $2^*|S_{CD}|=1$ stands for ordered systems.

2.10. Sperm cell labelling with fluorescent lipids

400 μg of BODIPY-PC powder was hydrated at 95% in SXN ($c=53$ mg/ml) by three freeze–thaw cycles at 50 °C. 200 μl of washed sperm (260 nmol phospholipids) were incubated with 4 μl of BODIPY-PC (260 nmol) for 1 h at 40 °C, centrifuged at 500×*g* for 15 min and washed twice in SXN. The sperm pellet was resuspended in SXN and incubated for 20 h at 40 °C to mimic the NMR experiments. BODIPY-PC-stained sperm were viewed under a 100× oil-immersion objective. BODIPY-PC was excited at 480 nm using a mercury source combined with an excitation filter HQ480/20 nm. The emission was detected by a dichroic beam-splitter and a HQ510/20 nm emission filter. The images were acquired on a Zeiss axiovert epifluorescence microscope with an Orca camera.

2.11. Quantification of incorporated deuterium-labelled lipids, in natural membranes by HPLC-electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS/MS)

Sperm membrane and MV0 were extracted after incubation with the deuterated lipid as described previously. Prior to extraction, 2 μg of each of the following internal standards (12:0/12:0 PtdCho, phosphatidylethanolamine (PtdEth), phosphatidic acid (PtdAc), Phosphatidylserine (PtdSer) and 16:0/16:0 phosphatidylinositol (PtdIns)) were added to the sample in an acidified chloroform/methanol (2.5:1) solvent. The extracted lipids were resuspended in chloroform/methanol/water (90:9.5:0.5) and characterised by tandem mass spectrometry (MS/MS) (API 3000, Sciex/Applied Biosystems) coupled to HPLC (series 200 micropumps, Perkin Elmer) [1]. For chromatographic separation a Luna silica column (1 mm×150 mm; Phenomenex) was used. The solvent gradient was 100% chloroform/methanol/water/ethylamine (90:9.5:0.5:7 mM) that transferred to 70% acetonitrile/chloroform/methanol/water/ethylamine (30:30:35:5:10 mM) at 20 min with a flow rate of 100 μl/min. PtdAc, PtdEth, PtdIns and PtdSer species were fragmented in the negative electrospray ionisation (ESI) mode (300 °C, −4 kV) and the precursor ions or neutral loss (nl) were 153 *m/z* (collision energy (CE): −40 V), 196 *m/z* (CE: −45 V), 241 *m/z* (CE: −60 V), nl 87 *m/z* (CE: −35 V). PtdCho species were fragmented in positive ESI mode (300 °C, +4 kV) and the characteristic fragment was 184 *m/z* (CE: +52 V). The retention time of the deuterium-labelled lipid was 0.5 min longer than their *m/z* charge non-deuterated equivalent lipid (Fig. 2).

3. Results

To demonstrate the generic application of our method we chose samples with different structural complexity. The method was applied to sperm cells and nuclear envelope precursor membrane vesicles (MV0) from sea urchin fertilised cytoplasmic egg extracts. High-resolution electron microscopy images (Fig. 1c) of sperm showed the complex network of mitochondrial membranes (M) that constitute most of the sperm membrane surface. The other major membranes were the nuclear envelope and the plasma membrane. Membrane vesicles were heterogeneous in size with vesicle diameters varying from 100 nm to 500 nm (Fig. 1a and b). Our main aim was to measure membrane dynamics by ^{2}H-NMR of these two opposing biological membranes after incorporating MLV or SUV of DPPC-$^2H_{62}$ and POPC-$^2H_{31}$.

3.1. Quantification of lipid probe incorporation in sperm and MV0 by HPLC-ESI-MS/MS

In order to optimise the conditions for probe incorporation, three important factors were taken into account: the ratio of probe to natural lipids in the samples, the centrifugation speed to remove excess probe and the vesicular dimensions of the probe to facilitate lipid incorporation. To optimise these experiments DPPC-$^2H_{62}$ was initially used since it was a more sensitive probe than deuterated POPC.

Samples were equilibrated at 40 °C for 30 min before centrifugation. After lipid extraction, phospholipids (PtdCho, PtdEth, PtdIns, PtdAc and PtdSer) were quantified by HPLC-ESI-MS/MS and cholesterol was quantified by colorimetric assay. A typical chromatogram is shown in Fig. 2. The chromatogram represents the separation of PtdCho species detected as $[M+H-184]^+$ ions in MV0 after incubation with DPPC SUVs. Natural PtdCho from MV0 eluted first. The elution peak was between 8.3 and 9.3 min. DPPC-$^2H_{62}$ eluted later between 9.5 and 9.9 min. The internal standard (12:0/12:0 PtdCho) eluted between 10.2 and 10.6 min. The probe to phospholipid ratio was calculated by comparing the natural phospholipid peak area with the deuterated-lipid peak area. Table 1 summarises the various parameters and the optimum ratio of DPPC-$^2H_{62}$ to natural membrane used. The recovery of biological membranes was estimated by comparing phospholipids and cholesterol measured in the supernatant with the same lipids measured in the washed pellet. Table 1 shows that addition of excess probe resulted in an inappropriate probe to natural lipid ratio (7–9:1). For labelling MV0, the best results were obtained with SUVs and elevated centrifugation speeds. The final optimum ratio of SUV probe to MV0 was 0.2:1. For labelling sperm, MLVs and moderate centrifugation speeds were used and these led to a high

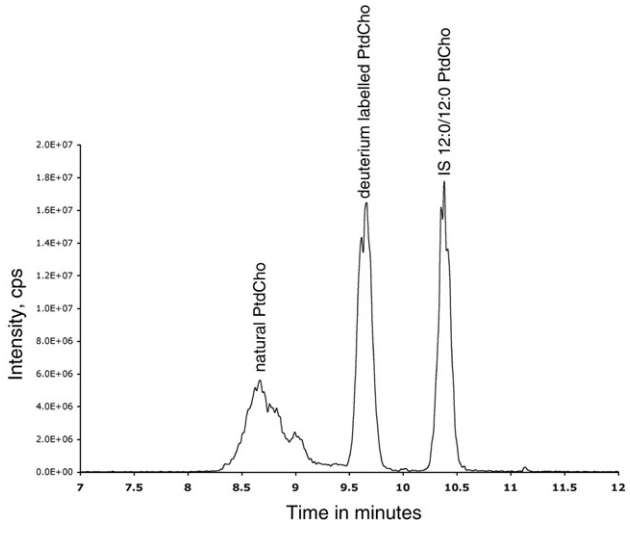

Fig. 2. HPLC chromatogram of lipid extract after incubation of MV0 with deuterium labelled DPPC-$^2H_{62}$ SUV. PtdCho was detected as $[M+H-184]^+$ ions using tandem mass spectrometry coupled to HPLC. 12:0/12:0 PtdCho was used as internal standard and added prior to extraction. Natural PtdCho was eluted first between 7.6 and 8.7 min and deuterium labelled DPPC-$^2H_{62}$ between 8.8 and 9.1 min.

membrane recovery. The optimal ratio of MLV probe to sperm membrane was 0.5:1. Note however that cardiolipin was not quantified in the native sperm lipids.

3.2. Solid-state ^{2}H-NMR of sperm and MV0 membranes using DPPC-$^2H_{62}$

As mentioned above DPPC-$^2H_{62}$ was initially used to optimise the experimental conditions. Fig. 3 (left panel) illustrates the spectra of sperm labelled with a 0.5:1 ratio of DPPC to natural lipids, at different temperatures. A spectrum was first acquired for 15 h at 20 °C and the temperature was augmented to 40 °C where a spectrum was also obtained. The temperature was lowered again to 20 °C to acquire a new spectrum (arrows in Fig. 3a). The equilibrium time between each temperature prior to each acquisition was 45 min. Due to the low quantity of deuterated material (0.96 mg or 1.2 μmol) acquisition times were relatively long. Fig. 3a (top spectrum) is a "powder" or non-oriented spectrum of ca. 140 kHz maximum width. The spectrum resembles a bilayer membrane in an ordered gel state. For comparison, Fig. 3b (top spectrum) shows the reference spectrum of pure DPPC liposomes at 20 °C, where it is in the gel state [29]. Both natural and model membrane spectra show similar, though not super imposable profiles. A minor isotropic peak was observed on the sperm spectra, which represented less than 5% of the total spectrum. This probably corresponded to the ^2H_2O natural abundance trapped in the vesicles that could not be totally removed during the preparation procedure. When the spectra were acquired at 40 °C (Fig. 3a, middle panel), a narrower spectrum of 50 kHz maximum width was observed with a profile characteristic of a lipid with axial symmetry. This was representative of a lamellar phase in the fluid state. The quadrupolar splitting ($\Delta\nu_Q$) of the methyl group at the chain end ($\Delta\nu_Q$ end) was measured to be 3.8 kHz. This narrow splitting was due to the high degree of free rotation of the carbon-deuterium bonds at the end of the fatty acid chain. The largest quadrupolar splitting, 29.3 kHz, measured on the spectrum corresponded to the most dynamically restricted carbon-deuterium bonds located near the interface (carbon positions 2–10), i.e. close to the phospholipid glycerol backbone. These dynamically equivalent carbon deuterium bonds are known as the "plateau" positions and the corresponding quadru-

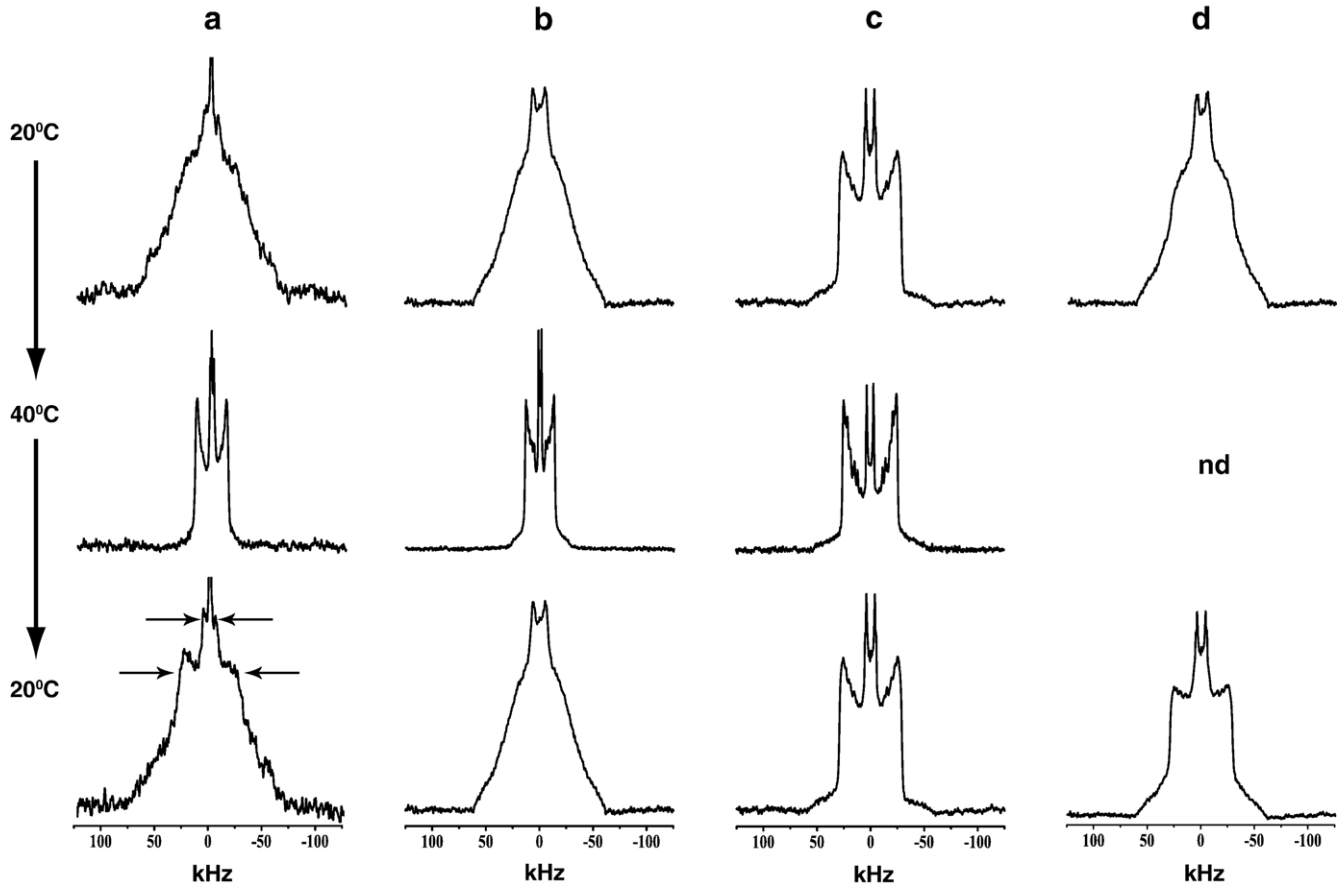

Fig. 3. Deuterium solid-state NMR spectra of DPPC-$^2H_{62}$ labelled sperm membranes and liposomes. The spectra of the first and last lines were acquired at 20 °C and the middle spectra at 40 °C. (a) Sperm membranes were incubated with DPPC MLVs for 30 min at 40 °C. The deuterium ssNMR spectra of labelled sperm membranes were acquired at 20 °C (top), at 40 °C (middle) and lowered back to 20 °C (bottom). The bottom spectrum profile differs from the top one in that some axial symmetry is observed (see spectrum shoulders) and the methyl and plateau quadrupolar splittings are resolved (arrows). Deuterium ssNMR spectra of DPPC liposomes containing 0 mol% (b) and 30 mol% cholesterol (c). Spectra from b and c were combined in d to match the spectra profiles in a. The top spectrum column d was obtained using a 0.8–0.9 fraction of pure DPPC spectrum and a 0.1–0.2 fraction of DPPC spectrum containing 30 mol% cholesterol. The bottom spectrum column d was obtained using a 0.4 fraction of pure DPPC spectrum and a 0.6 fraction of DPPC spectrum containing 30 mol% cholesterol.

Table 2
Cholesterol content, "plateau" quadrupolar splittings ($\Delta\nu_Q$) and order parameters ($2S_{CD}$) for deuterium labelled DPPC and POPC incorporated in MLVs and in intact cell membranes

Sample	Cholesterol content (%) [a]	Temperature	DPPC		POPC	
			$\Delta\nu_Q$	$2S_{CD}$	$\Delta\nu_Q$	$2S_{CD}$
MLV	0	10 °C	nd	nd	28.9	0.458
		20 °C	nd	nd	26.7	0.426
		40 °C	26.8	0.420	23.5	0.375
	5	10 °C	nd	nd	32.2	0.514
		20 °C	nd	nd	29.2	0.466
		40 °C	32.0	0.511	24.8	0.396
	15	10 °C	nd	nd	35.9	0.573
		20 °C	nd	nd	32.4	0.517
		40 °C	35.4	0.568	27.4	0.437
	30	10 °C	nd	nd	45.0	0.718
		20 °C	57.4	0.910	40.7	0.650
		40 °C	50.4	0.800	34.0	0.543
Sperm	29±1	T_{ini}	nd	nd	30.7	0.49
		40 °C	29.3	0.46	23.5	0.37
		T_{fin}	55.0	0.87	34.0	0.54
MV0	43±2	T_{ini}	nd	nd	31.5	0.50
		40 °C	37.0	0.59	25.0	0.40
		T_{fin}	50.0	0.79	37.6	0.60

Deuterium spectra of labelled sperm and MV0 were acquired at low temperature (T_{ini}), increased to 40 °C and lowered back to a final temperature (T_{fin}). T_{ini} and T_{fin} refer to either 20 °C for DPPC values or 10 °C for POPC values.
nd: not determined.
[a] Mol cholesterol percentages were estimated from colorimetric assay quantification.

polar splitting is $\Delta\nu_Q$ plat. Other labelled positions could not be resolved easily. For comparison, the spectrum of pure DPPC liposomes at 40 °C is shown in Fig. 3b, middle panel. The $\Delta\nu_Q$ end=3.2 kHz and $\Delta\nu_Q$ plat=26.8 kHz were measured. These values were markedly lower than those obtained on the lipid probe embedded in sperm membranes. Fig. 3a (bottom panel) the sample was cooled down to 20 °C. A "powder" spectrum was again detected with a spectral profile significantly different from the one initially observed at 20 °C. A fraction with axial symmetry was clearly seen and the methyl group splitting (arrows) was detected to be ca. 12 kHz. A "plateau" like splitting of ca. 55 kHz could also be measured (arrows). This indicated that the initial spectrum acquired at 20 °C was not at equilibrium and it was necessary to incubate the system at 40 °C to increase the lipid incorporation into the sperm membranes. The sperm spectra were more ordered (Table 2) than pure DPPC-$^2H_{62}$ liposomes at 40 °C and showed an axially symmetric characteristic when the temperature was lowered to 20 °C ($2*S_{CD}$ close to 0.9). This suggested that an ordering promoter such as cholesterol could be implicated. To verify this possibility, control experiments were performed with liposomes containing 30 mol% cholesterol (Fig. 3c). The measured quadrupolar splittings were $\Delta\nu_Q$ end=8.3 and 6.42 kHz and $\Delta\nu_Q$ plat=57.4 and 50.5 kHz at 20 °C and 40 °C, respectively. These values were in agreement with previous studies [6,30,31].

To further investigate the sperm spectrum acquired at the final temperature of 20 °C the sperm spectral profile was reconstructed from the control DPPC spectra with and without 30 mol % cholesterol (Fig. 3d). This type of comparison could be made since NMR is a precise quantitative method. The top spectrum (Fig. 3d) was generated using the following linear combination: (0.8 to 0.9)*(DPPC spectrum, Fig. 3b)+(0.1 to 0.2)*(DPPC 30 mol% cholesterol spectrum, Fig. 3c). The bottom spectrum was obtained, in the same manner, using a 0.4 fraction of pure DPPC spectrum (Fig. 3b) plus a 0.6 fraction of DPPC 30 mol% cholesterol spectrum (Fig. 3c). Although an approximation, these linear combinations showed that the spectra in Fig. 3d resembled those of Fig. 3a. The purpose of these spectral combinations was to demonstrate that the lipid probe sensed highly ordered environments that are usually induced by cholesterol in membranes [15]. Therefore, this illustrated that DPPC was not fully incorporated at the initial 20 °C. Further incubation at 40 °C was necessary to reach equilibrium before lowering to the final 20 °C where the deuterated DPPC was exchanging slowly (μs) with different environments.

Similar experiments were performed with MV0. Fig. 4a shows the deuterium spectra obtained with 1 mg of DPPC SUVs incubated with MV0 at the initial temperature. Spectra were acquired with only 300 μg or 375 nmol of labelled lipids (Table 1), which resulted in a low signal-to-noise ratio even after 30 h of acquisition. The same temperature sequence as sperm samples was used (Fig. 3a). In Fig. 4a (top, middle and bottom spectra) all spectra show a super imposition of a narrow and intense line with powder patterns. The intense narrow peak (50% of the total intensity Fig. 4a top panel) was attributed to ^2H_2O natural abundance and non-fused SUVs undergoing isotropic motion. Fig. 4b is the control where the isotropic sharp line of pure SUVs is shown for the corresponding temperature sequence. This panel also demonstrated that SUVs did not fuse to each other during the experiment. Quadrupolar splittings measured for plateau positions at 40 °C and 20 °C (arrows in Fig. 4a middle and bottom panels) were approximately 37 and 50 kHz respectively. In the DPPC control experiments, the "plateau" quadrupolar splittings were 26.8 and 50.4 kHz for pure and cholesterol-containing membranes at 40 °C and 57.4 kHz for cholesterol-containing membranes at 20 °C (Table 2). DPPC probed more ordered membranes in MV0. Table 2 shows the order parameter at the final 20 °C was 0.8 suggesting the presence of cholesterol in MV0. However, in these experiments the *S/N* ratio was too low to allow a spectral linear combination as performed in Fig. 3.

3.3. Sperm and MV0 membrane dynamics probed by deuterium solid-state NMR using POPC-$^2H_{31}$

To assess membrane dynamics closer to physiological conditions we used an unsaturated neutral lipid probe that would not affect membranes that already have more than 50 mol % polyunsaturated phosphatidylcholine. Deuterium-labelled 16:0($^2H_{31}$)/18:1 PtdCho (POPC-$^2H_{31}$) was used to assess the dynamics of sperm and MV0. It is intrinsically more fluid because of its low transition temperature at −4 °C [32,33]. To label the samples with POPC the same procedure as in the case of DPPC was used. Sperm cells (3.8 mg or 4.7 μmol) were incubated with 4.7 μmol of POPC MLVs for 30 min at 40 °C. Fig. 5a shows the deuterium spectra of labelled sperm acquired

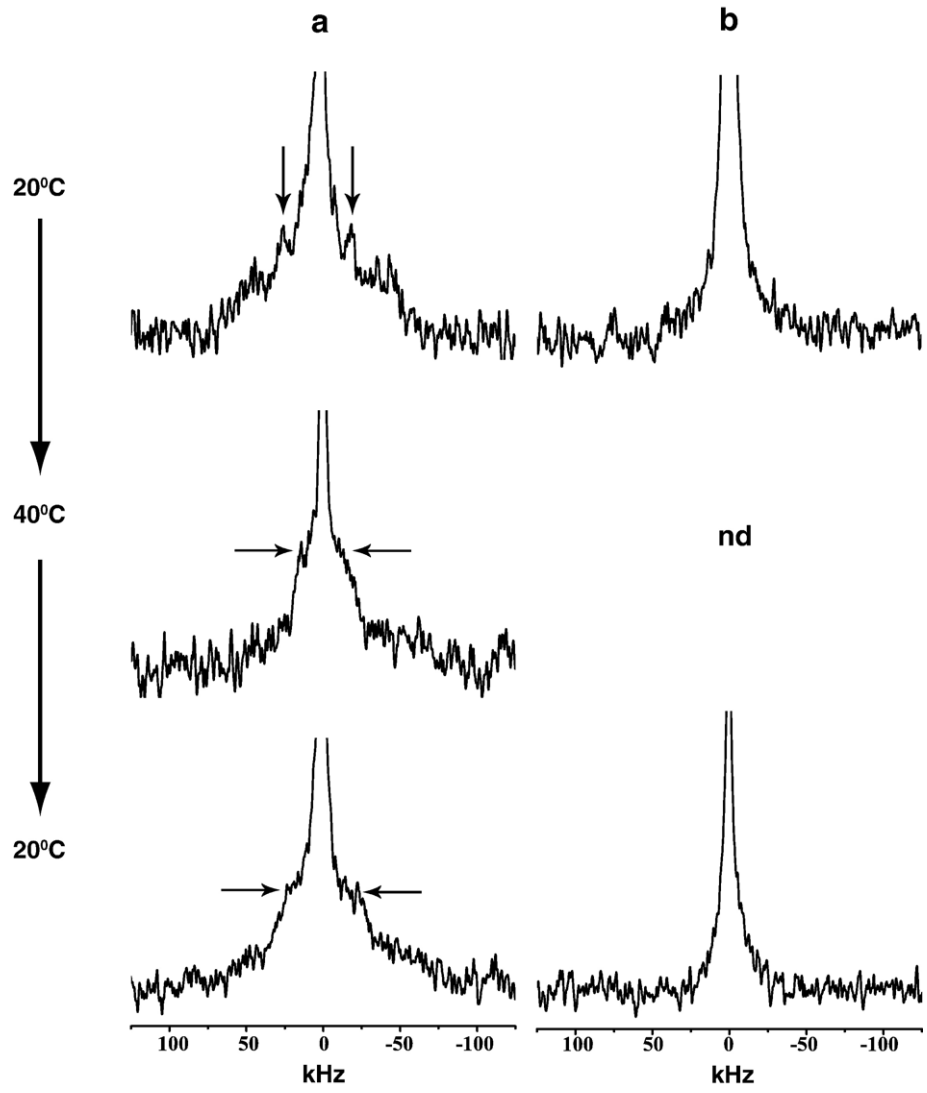

Fig. 4. Deuterium solid-state NMR spectra of DPPC-$^2H_{62}$ labelled MV0 and liposomes. (a) MV0 was labelled with DPPC SUVs for 30 min at 40 °C and the spectra acquired at 20 °C (top), at 40 °C (middle) and lowered to 20 °C (bottom). (b) The second column displays SUVs of DPPC spectra acquired at 20 °C before (top) and after (bottom) equilibrium at 40 °C.

at T_{ini} = 10 °C, just after sample preparation (middle panel) and at T_{fin} = 10 °C, after the temperature was increased to 40 °C (bottom panel). The low temperatures were chosen since these were close to the physiological environment of the sea urchin. The POPC-MLV control experiments (top spectrum) showed a "powder" spectrum typical of a lipid in a fluid lamellar phase. The methyl and plateau quadrupolar splittings were 3.6 and 28.9 kHz wide respectively. The middle spectrum was also characteristic of a probe in an axially symmetric environment typical of a fluid-like lamellar phase. The isotropic peak was cropped to show more clearly the powder pattern. This sharp peak represented about 10% of the total area and could be attributed to ^2H_2O natural abundance. The values for $\Delta\nu_Q$ plat and $\Delta\nu_Q$ end were 30.7 kHz and 4 kHz respectively. Measurements of the methyl quadrupolar splitting were more difficult due to the isotropic peak. The same comments can be made for the bottom spectrum, acquired after further incubation for 20 h at 40°C. In this case larger splittings were measured: $\Delta\nu_Q$ plat = 34 kHz and $\Delta\nu_Q$ end = 4.5 kHz. These greater values suggested elevated amounts of cholesterol. Control experiments with variable amounts of cholesterol in POPC MLVs were performed (spectra not shown) to enable the comparison of these data with model membranes. Table 2 indicates the quadrupolar splittings for "plateau" positions and their corresponding order parameters. The order parameters were comparable to those obtained from POPC-labelled sperm.

MV0 was incubated with 8.25 μmol of POPC-$^2H_{31}$ SUVs for 30 min at 40 °C (Materials and methods). Fig. 5b illustrates the POPC deuterium spectra at T_{ini} = 10 °C, just after sample preparation (middle panel) and at T_{fin} = 10 °C, after raising the temperature to 40 °C (bottom panel). The MLV control is shown in Fig. 5a (top panel). The middle and bottom spectra are again characteristic of lipids in a fluid-like lamellar phase. The isotropic peak was cropped to illustrate the powder pattern. The

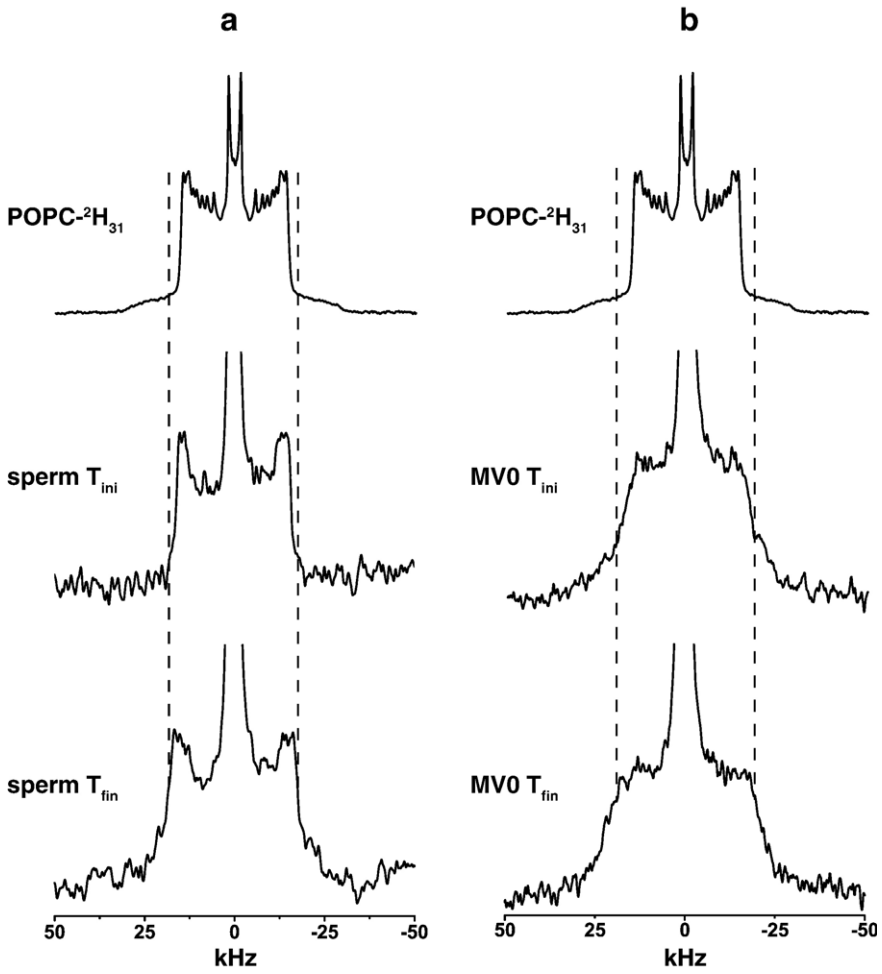

Fig. 5. Deuterium solid-state NMR spectra of POPC-$^2H_{31}$ labelled sperm membranes (a), MV0 (b) and liposomes (a and b). Sperm (a) and MV0 (b) were labelled with MLVs and SUVs of POPC respectively for 30 min at 40 °C and the corresponding deuterium NMR spectra acquired at 10 °C (middle spectra: «sperm T_{in}», «MV0 T_{ini}»). The systems were equilibrated at 40 °C and reacquired at 10 °C (bottom spectra «sperm T_{fin}», «MV0 T_{fin}»). The top spectrum corresponds to MLVs of POPC and the dashed lines show the plateau quadrupolar splitting enlargement on sperm and MV0 spectra post equilibrium at 40 °C.

isotropic peak represented about 10–15% of the total area and was assigned to ^2H_2O natural abundance or to non-fused SUVs. It is of note that this line is much more reduced than with the DPPC probe indicating that POPC fused better with the cell membranes. The methyl quadrupolar splittings were not well resolved and the plateau quadrupolar splittings were 31.5 kHz for the middle spectrum (T_{ini} = 10 °C) and 37.6 kHz for the bottom spectrum (T_{fin} = 10 °C). Comparison with control data on cholesterol-containing POPC MLVs suggests that cholesterol may promote such an ordering effect.

3.4. Membrane integrity after NMR experiments and colorimetric quantification of cholesterol

To ensure the integrity of the sperm cell membranes after incubating the sperm at 40 °C for 20 h with the probes, the samples were separately incubated with BODIPY-PC MLVs, and fluorescent images were acquired to see if the morphology of the sperm cell was modified. Fig. 6 shows that prior to and after equilibrium at 40 °C, fluorescent images were identical. The phase images also show the sperm morphology was not modified.

Quantification of total phospholipids and cholesterol in sperm membranes and MV0 was obtained by colorimetric assays (see Materials and methods). The quantity of cholesterol was normalised to the total phospholipids. Sperm and MV0 contained 29.2 ± 1.4 mol% and 43.1 ± 1.7 mol% cholesterol respectively.

4. Discussion

The development of less invasive methods for measuring the physical properties of membranes would be essential for directly relating membrane structural properties to their biological function. Therefore, our objective was to develop a method where membrane dynamics in intact cells or purified subcellular membrane compartments could be determined directly. For the first time deuterium solid-state NMR spectroscopy was exploited to probe the dynamics of sperm cells and nuclear envelope precursor membrane vesicles. The outcome of our

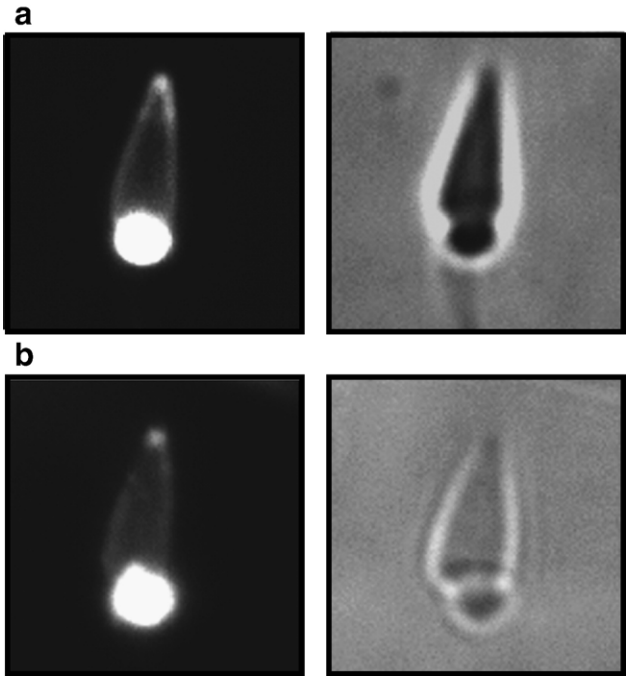

Fig. 6. Sperm membrane fluorescent imaging after high temperature treatment. Sperm cells were incubated with BODIPY-PC MLVs for 1 h at 40 °C, washed twice at 500 g for 15 min and incubated for 20 h at 40 °C. BODIPY staining was monitored prior to (a) and post (b) 20 h incubation at 40 °C. BODIPY-PC was excited at 480 nm. The labelling was very similar prior to (a) and post (b) incubation. BODIPY-PC labels sperm plasma membrane, mitochondria and nuclear envelope. Bars are 250 nm.

studies was that both sperm and nuclear envelope precursor membrane vesicles were ordered membranes due to their high levels of cholesterol. The order parameter of the latter was higher due to its relative elevated levels of cholesterol.

4.1. Membrane dynamics of intact cells are probed less invasively

To probe the dynamics of cell membranes less invasively two factors need to be taken into consideration. First, the methodology should not perturb the membrane structure and second the probe should not effect the lipid composition so that the membrane structure would be not modified. The first question is addressed intrinsinctly as NMR is non-invasive. To address the second issue the type of probe and the quantity required to label the samples needed to be optimised.

We used both deuterated DPPC and POPC. DPPC was essentially used for optimisation of the initial NMR experiments. On DPPC, both fatty acid chains were deuterated and had twice as much signal than POPC that only had one deuterated chain. DPPC had a gel-to-fluid transition temperature close to 40 °C whereas POPC was in the fluid phase above −4 °C [32,33]. For proper incorporation of DPPC the sample needed to be at 40 °C for a relatively long time. However, due to the unsaturated fatty acid chain of POPC and its fluid state at lower temperatures it incorporated much faster. The variations in temperature that were used in this study were based on the physiological temperature of the sea urchin that is between 10 and 20 °C. At this range of temperature, pure DPPC is in the gel phase and in a liquid-ordered phase when in contact with cholesterol enriched domains [15,34–36]. Spectral combinations were required to obtain accurate order parameters from the complex spectral profiles. However, with POPC, deuterium spectra were axially symmetric and the order parameter was easily obtained from the quadrupolar splitting measurements. The latter is one of the main reasons for using POPC as the preferred probe even though it has less deuterium signal than DPPC. Furthermore, sea urchin sperm and nuclear membranes contain mainly polyunsaturated lipids [1,37], thus POPC is a more physiological probe and affects less the native structure of the membrane.

We determined the optimal probe-to-natural lipid ratio to be between 0.2:1 and 0.5:1. These ratios were obtained from the quantification of phospholipids (PtdCho, PtdIns, PtdEth, PtdSer, PtdAc and PtdGly) and cholesterol in natural membranes. However, inner mitochondrial membranes contain high amounts of cardiolipin [38]. In bovin heart for example, 20 mol% of mitochondrial membranes are cardiolipin lipids [39]. Assuming that mitochondrial membranes in sea urchin sperm represent more than 80% of the total membranes (Fig. 6) and have a similar composition to mammals, the real probe-to-lipid ratio in labelled sperm would be less than 0.48:1. So the ratio we report is the maximum ratio based on the phospholipids we analysed. Moreover, natural membranes also contain integral proteins that represent half to two third of the mass of the membrane. Therefore, the amount of lipid probe in the membrane could easily go down to 3–10%. Sperm membranes and MV0 phospholipids contain 40 to 50 mol% of unsaturated phosphatidylcholine [1] (Garnier-Lhommet et al., manuscript in preparation 2007) therefore the addition of a lipid with a neutral curvature such as POPC should not effect their structural properties. Finally, the form, which the probe was introduced to the target membrane, was also important. In general, if intact cells (few microns) were to be used, large liposomes (MLVs) should be utilised. The incorporation of MLVs is demonstrated by the fluorescent images (Fig. 6) that show MLVs incorporated readily in sperm cells after a long incubation at 40 °C. The first incubation of 30 min at 40 °C allowed probe attachment to natural membranes but fusion of the probe with membrane occurred only during long incubations at 40 °C (>10 h). Small liposomes (SUVs) were not used to label sperm cells since under mild centrifugation conditions sufficient amounts of SUVs were not pulled down for adequate labelling. To label purified subcellular membrane compartments or membrane vesicles such as MV0 (approximately 400 nm) small liposomes were used. Large MLVs did not fuse with MV0.

In summary the probe needs to be added to the target membrane as vesicles that have the same or smaller dimensions as the cell or organelle under investigation. Moreover, to overcome the lower sensitivity of POPC compared to DPPC larger sample volumes and higher magnetic fields can be used. Our experiments were mainly performed at 12 T. Using spectrometers that operate at 17–24 T would improve the sensitivity. In the future cryo-probes that may be implemented for solid state NMR could increase the signal-to-noise ratio by at least four

orders of magnitude and augment the detection limit so that even less probe would be used to label natural intact membranes.

4.2. Membrane order in intact cells and MV0 is linked to their cholesterol content

In our study, at 10 and 20 °C, we measured an elevated membrane order parameter that may be linked to high amounts of cholesterol both in sperm and MV0. Determination of cholesterol content by colorimetric assays indicates that MV0 contained more cholesterol (43%) than sperm membrane (29%). From the NMR spectra we determined the order parameter of MV0 ($2*S_{CD}=0.60$) to be 10% more than sperm ($2*S_{CD}=0.54$—Table 2). Therefore the dynamics measured in situ correlates with the higher cholesterol content of MV0 compared to sperm. If the order parameter of MV0 was directly compared to model membranes containing cholesterol (Table 2) it would correspond to MLVs with less than 30 mol% cholesterol. It should be noted that this slight discrepancy may be due to components of the membrane, other than cholesterol, that modulate dynamics. Additionally, with the POPC probe only average order parameter values were determined as the low signal-to-noise ratio prevented detection of membranes with variable dynamics. Furthermore, MV0 is an egg extract which intrinsically contains high levels of cholesterol [40], therefore it is not surprising that the probe does not sense membrane areas with different levels of cholesterol. Due to the lower intrinsic cholesterol content of sperm cells, it could be suggested that the DPPC probe sensed regions with high and low cholesterol. Fluorescent images (Fig. 6) show that the fluorescent probe incorporated into all the membranes including the mitochondrial membrane that constitutes the main membrane surface. While the exact amount of cholesterol in non-somatic mitochondria is unknown, mitochondrial membranes in somatic cells have less than 3 mol% cholesterol [41]. We can therefore extrapolate that the DPPC probe incorporated into both the plasma membrane, with high levels of cholesterol, as well as the mitochondrial membrane that may contain lower quantities of cholesterol.

In conclusion our new application can be exploited as a generic method for monitoring membrane dynamics in whole cells and various subcellular membrane compartments.

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